THE POSSIBLE RELATIONSHIP BETWEEN BACTERIA AND THE GUM OF *HAKEA SALIGNA*.

(Bac. pseudarabinus ii., n.sp.)

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Specimens of gum that were picked from *Hakea saligna* appeared as transparent, colourless, rounded and conical masses as well as clusters of conical drops (tears). Other fragments were brownish-yellow and blackish. They were easily cut with a knife, showing that they had a tough and gelatinous but not a brittle consistency. Some of the conical masses measured about 4 cm. in circumference and 1 cm. high, the base being attached to the bark of the tree through punctures from which the gum had exuded.

The gums of species of *Hakea* other than *Hakea saligna* have been examined by Maiden,* who found that they consisted of arabin (5-16 %) and pararabin (63-69 %), together with moisture and ash. He also notes† the presence of a jelly at the roots of certain Hakeas, but that was probably the undried gum.

The gum which I received when placed in a small quantity of water swelled in it, forming a thick paste of the lumpy consistency of fruit jam. With much water a homogeneous paste was formed in a day or two. The water made no differentiation between parts of the gum; it appeared to be a single substance, not a mixture of two constituents with differing solubilities. The viscosity of the gum appeared to be midway between arabin and metarabin.

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* Proc. Roy. Soc. S. Australia, 1889, 54.

† Proc. Roy. Soc. N. S. Wales, 1901, 161.

The examination, by means of plate cultures, of the tissues of branches near the site of the exudate, revealed the presence of a number of bacteria, many of which appeared to be capable of producing gum. Some of the original colonies were slimy, ropy or gelatinous and did not liquefy the gelatine, while others liquefied the glucose-gelatine upon which they were growing. Many of those colonies which were considered to be representative were picked out and grown upon saccharose-potato-agar, upon ordinary nutrient agar and in saccharose-peptone-fluid.

These secondary cultures enabled the bacteria to be classified into races of several probable species. Those bacteria which gave no evidence of being capable of producing slime readily upon the media already mentioned as well as upon levulose-asparagine-tannin-agar* were rejected as being of no importance. Typical growths of the several supposed species were "plated" in glucose-gelatine to ensure the purity of the bacteria.

Two bacteria were recognised as races of *Bac. levaniformans;* one of them grew as a thin wrinkled skin on saccharose-potato-agar,† while the other produced a luxuriant semi-white slime. Eventually the probable formers of *Hakea* gum were reduced to two, one of which readily produced a ropy and almost transparent slime in saccharose-asparagine-fluid.‡ On levulose-asparagine-tannin-agar the slime was cohesive and suggestive of a gum, the solubility of which was similar to that of the natural gum. The other will be referred to subsequently.

The colonies upon glucose-gelatine were like little whitish irregular masses, which appeared semi-opaque with irregularly darkened patches when viewed microscopically. The stroke on glucose-gelatine appeared as a broad, whitish, wrinkled ribbon with a depressed centre. The gelatine was at first consumed as was shown by the depression in the middle of the growth; after-

* Levulose 20, asparagine 1, tannin 1, potassium citrate 1, agar 20 grm., water to 1000 c.c.

† Potato juice 250, glycerine 10, saccharose 20, agar 20 grm., water to 1000 c.c.

‡ Saccha ose 50, asparagine 1, potassium citrate 5, tap-water 1000 cc.

wards it became liquid. The stab-culture in the same medium developed as a rough thread at the top of which stood a wrinkled, semi-transparent, whitish nail-head. The upper layers of the gelatine became liquefied in a stratiform manner. Upon nutrient agar the culture was at first narrow and dirty-white or yellowish and raised with a slightly wrinkled flat margin. The growth became broader and the wrinkles more pronounced, while the centre deepened in colour to buff. The potato-culture was buff, raised and dry. Bouillon became very turbid, producing a film and a coherent sediment. The indol reaction was obtained and nitrates were reduced to nitrites. Milk became slightly viscous. On saccharose-potato-agar the growth was luxuriant, convex, slimy and dirty-white; the margin was flattened and rough and the condensed water became a thick slime. The organisms generally exhibited polar staining, and they were negative to Gram's method. The cells, which were motile, varied in size. In bouillon they ranged from $0{\cdot}5 : 0{\cdot}7\,\mu$ to $0{\cdot}75 : 1{\cdot}5\,\mu$, but generally measured $0{\cdot}6 : 1{\cdot}2\,\mu$. On saccharose-potato-agar, they varied from $0{\cdot}3 : 0{\cdot}8\,\mu$ to $0{\cdot}7 : 1{\cdot}2\,\mu$.

The nature of the colonies on glucose-gelatine (indicative of the production of an insoluble gum), as well as the slow liquefaction of the medium, show that the bacterium has its nearest allies in *Bact. metarabinum* and *Bac. atherstonei*.

A quantity of the slime was prepared by growing the organism on a medium containing saccharose 20, potato-juice 100, glycerine 10, tannin 3, and agar 20 grm. in the litre. A prescription almost similar had been used originally in growing the slime of *Bact. metarabinum*, but it had been subsequently discarded, as no slime could be obtained upon it. The slime tended to adhere to the agar, but this was overcome by pouring about 10 c.c. of a nutritive fluid over the growth in each plate. The nutritive fluid consisted of the levulose-asparagine fluid, the prescription for which has already been given, and it was used because it chanced to be convenient at the time. The slime yielded a gum by the autoclave treatment. This gum, when dehydrated, absorbed water, becoming a thick mucilage. It did not dissolve

freely like the arabin from *Bact. acaciæ*, but seemed to swell like the natural gum of *Hakea*. The mucilage was tested, and it gave the reactions for arabin, that is to say, it was coagulated by alcohol, basic and ammoniacal lead acetates, by ferric chloride. Fehling's solution gave no precipitate, although 1 % copper sulphate followed by potassium hydrate gave a colourless coagulum. Barium hydrate gave a slight precipitate, while other reagents were inactive.

When hydrolysed by boiling with 5 % sulphuric acid for five hours, the gum yielded reducing sugars, and these were readily recognised as arabinose and galactose by means of their osazones.

Thus the organism was a race of *Bact. acaciæ*, midway between that bacterium which produces arabin and its modified form which gives metarabin, and which I have named *Bact. metarabinum*. Although the cultural characters were not typical of either of the forms, yet the points of variance were not sufficiently pronounced to say that it was not *Bact. metarabinum*. the diagnosis of the gum bacteria is not an easy matter, because the cultural characters of some of the races are extremely variable. Slight alterations in the chemical constitution of the gum influence its degree of solubility, and that controls the microscopical appearance of the colony. The colour is another point upon which too much reliance cannot be placed. That has been emphasised by my researches upon *Bac. levaniformans* and upon *Bac. pseudarabinus*, both of which occur as white or as yellow races.

Meanwhile the natural gum of *Hakea saligna* had been under investigation to see if it was really a variety of metarabin. The swollen gum was useless for the purpose of testing with reagents, as the drop of mucilage was too gelatinous to mix with the drop of reagent. By subjecting the gelatinous suspension of the swollen gum to a pressure of three atmospheres in the autoclave, a thin solution of a soluble modification was obtained, and upon evaporation this yielded a good mucilage which was tested with several reagents. Basic and ammoniacal lead acetates gave transparent clots. Barium hydrate gave a white precipitate, but with

another mucilage a thickening only was produced. Neutral lead acetate, ferric chloride, copper sulphate, silver nitrate, iodine, tannic acid, Fehling's and Schweitzer's solutions gave no reactions. The hydrates of some of the metals formed insoluble compounds with the gum. Ferric chloride followed by a trace of potassium hydrate gave a transparent yellow clot; barium chloride and alkali produced a mottled transparent and white clot; copper sulphate gave a transparent coagulum. Potassium hydrate thickened the mucilage. These are not quite the reactions either of arabin or of soluble metarabin. A coagulation with neutral 1 % ferric chloride is, as for as I know, always obtained with these mucilages. Attempts were made to induce a coagulation. As the gum was acid, the mucilage was neutralised and then dialysed to remove any organic salts that might possibly have prevented coagulation. Still no reaction was obtained. Other methods were tried, such as by making the gum neutral to litmus and precipitating with alcohol, but by no method could a positive reaction with ferric chloride be procured. The probability was that the gum was neither arabin nor metarabin.

The gum was hydrolysed by boiling it with 4 % sulphuric acid for four hours. The solution contained substances that reduced Fehling's solution, but no recognisable osazones could be obtained. With the same treatment, arabin and metarabin give arabinose and galactose, the osazones of which can be obtained and recognised with comparative ease; pararabin is not attacked by that strength of acid. The gum of Hakea, therefore, did not appear to contain any of these substances, so that it differed from the common vegetable gums. Since, however, reducing bodies resulted from the hydrolysis, it seemed advisable to repeat the operation with some alterations. These were the boiling of the acid solution for a shorter time and the use of a smaller amount of acetic acid in the phenylhydrazine mixture. In the course of many recent researches, the proportion of acetic acid had been increased until the mixture contained three parts of glacial acetic acid to one part of phenylhydrazine. The reason for this was that certain indefinite substances that simulated osazones

were destroyed by the acid and removed as a partly soluble oil or tar. But this will be seen more clearly as we proceed.

A second portion of the natural gum was boiled for one hour with 4 % sulphuric acid, neutralised with barium carbonate and precipitated with alcohol. The insoluble gum was boiled for an hour with 5 % acid. From this no gum precipitable by alcohol was obtained. Both these portions gave osazones with phenylhydrazine. By treatment with ether followed by water the precipitates were separated into portions which melted between 107° and 117° and into others which melted between 168° and 186°. In one of the latter portions, while the bulk melted at 186°, the dust in the tube melted at 190°, which suggested the possible presence of galactosazone. Its actual presence, however, could not be proved. The portions with the low melting points were largely destroyed when evaporated with dilute acetic acid.

In view of the unsatisfactory results which had been obtained, a third portion was hydrolysed. The gum was dissolved in the autoclave and coagulated with alcohol. The coagulum after being dissolved in water and evaporated, was tested with Fehling's solution; no reduction was obtained. The gum was boiled under an aerial condenser for an hour with 0·5 % sulphuric acid. The neutralised and evaporated solution was treated with alcohol and filtered. The insoluble gum was boiled with 1 % acid for an hour and treated as described. The gum unacted upon was boiled with 5 % acid for an hour, when only a trace of unattacked gum remained. Thus there were obtained three solutions of hydrolysed gum, and all reduced Fehling's solution. I shall describe the investigation of the products of hydrolysis with some detail because similar reducing substances were obtained from the gum of linseed mucilage. The reducing solutions were treated while on the water-bath with phenylhydrazine mixture containing of the base 1 part, glacial acetic acid 1 part, glacial acetic acid saturated with sodium acetate 1 part, water 1 part. The acetate was added because potassium chloride was sometimes used to flocculate the milky solutions of the gum. Any tarry matter that formed during the heating on the water-bath was

removed by filtration through a hot, wetted, double filter, and the osazones that formed upon the cooling of the solution were filtered off, dried on porous porcelain and examined. As a rule these osazones formed as voluminous yellow masses which consisted of interlacing, indefinite, feathery, crystalline tufts. Sometimes the mass appeared as a flabby yellow jelly, which shrank to small volume on the filter. The solutions were treated twice with phenylhydrazine solution, a third treatment being unnecessary. The individual precipitates were kept separate until the melting points showed that the fractions were similar.

The osazones of the first fraction of the first hydrolysis (0·5 % acid) dried on porcelain as brownish skins like arabinosazone, but they were largely soluble in ether, which enabled four fractions to be obtained; these melted at 118°, 118-120°, 122°, and 132°. They appeared to consist of the same osazone or mixture of osazones; the ethereal solutions upon evaporation left reddish-yellow crusts which, when dissolved in water and evaporated, gave a yellow amorphous powder and a reddish-brown vitreous film. Evaporation with dilute acetic acid converted everything into a brownish tar. The second fraction of the first hydrolysis was separated by ether into portions melting at 118°, 123-126°, 130°, and 142° with the same characters as the portions of the first fraction.

The fractions of the second hydrolysis (1 % acid for 1 hour) gave osazones which also dried on porcelain as brown skins, but which melted at 158-159° and at 164-166°. These were added together and treated with water on the water-bath. A part dissolved and separated out upon cooling as stellate clusters of delicate needles mixed with small jagged spheres. On porcelain, the precipitate formed a brown skin which melted at 114°. The residue was again heated with water and filtered. A precipitate of indefinite crystals separated out upon cooling. These melted between 162° and 166°. The residue, insoluble in the quantities of hot water that were used, was suspended in a small volume of water and carefully treated with alcohol while on the water-bath, until the whole of it dissolved. The alcoholic solution was cooled

when a yellow precipitate which melted at 176-178° separated out. A vitreous yellow residue was obtained upon evaporating the mother-liquor. When dissolved in water and evaporated, the same vitreous residue remained.

The third hydrolysis (5 % acid for 1 hour) yielded two fractions. The first was a brown powder which when treated with hot dilute acetic acid, to dissolve the brown constituent, furnished, upon cooling, rosettes of microscopic needles which melted at 130°. A dirty-brown vitreous residue was obtained upon evaporating the mother-liquor. The second osazone was a yellow powder which melted at 174-176°. Ether extracted a constituent which dried as reddish-yellow crusts and when evaporated from aqueous solution gave a slight yellow powder and a brown tar. Following the ether treatment, the yellow powder was heated with water upon the water-bath and filtered; the residue was again heated with water and filtered. Both hot solutions deposited, upon cooling, yellow precipitates of small microscopic spheres which dried as brown skins. The first melted at 162-164°; the second melted at 170°. The first was heated with water and filtered, when stellate tufts of *pale yellow needles melting at 162°* settled out. The residue, insoluble in the hot water, melted at 166°. The residue of the original yellow powder not dissolved by the repeated treatment with hot water was dissolved in alcohol, heated until much alcohol had evaporated, and then cooled. The osazone that settled out melted at 182°, while the residue obtained upon the evaporation of the mother-liquor melted at 178°. The osazone (182°) was treated with cold strong alcohol which dissolved a constituent melting at 179°, leaving undissolved *a lemon-yellow osazone melting at 186-187°*. An osazone with a melting point higher than this could not be obtained.

The italics in the last paragraph indicate the osazones which approached most closely to arabinosazone and galactosazone. Although they simulated these compounds, there can be no doubt that they were other substances, for it is a comparatively easy matter to separate the definite osazones of arabinose and galac-

tose. The compound that simulated galactosazone was much too easily soluble in water and in alcohol, while the arabinosazone-like body did not separate out from water with the normal appearance of that substance. It was difficult to say whether one had to do with interlacing crystals or with a jelly, and, further, the precipitate while on the filter was more gelatinous than the crystalline precipitate of arabinosazone. The osazone-like bodies with the low melting points (about 120°) were decomposed by acetic acid. It must, therefore, be concluded that arabinose and galactose are not among the products of hydrolysis, and furthermore that the osazones that are produced lack the definite characters of the osazones of the well-known sugars. The gum is hydrolysed to substances that reduce Fehling's solution, that give off the furfural odour during hydrolysis, but which give indefinite osazones with phenylhydrazine. The latter can be separated into groups which have melting points about 120°, 160° and 190°. But since dilute nitric acid oxidises the gum to mucic acid, it must be assumed that there is present a substance allied to a galactan. Possibly these indefinite bodies are akin to the furfuroïds which Cross, Bevan and Smith obtained from straw. It is also possible that the gum is that indefinite but much referred to substance, pectin.

It was evident from the investigation of the products of hydrolysis that the gum was not metarabin and that the bacterium allied to *Bact. metarabinum* probably played no direct part in its production. At the same time it must not be forgotten that *Bact. metarabinum* is capable of producing a secondary substance which while yielding arabinose and galactose upon hydrolysis, did not give the typical reactions for arabin or dissolved metarabin, inasmuch as it gave a precipitate with barium hydrate. I suggested that it was possibly a pectin body.

Since the organism did not appear to be responsible for the gum, I investigated the only other which produced a ropy solution in saccharose-asparagine-fluid. This grew as a thick, mottled-white slime on saccharose-potato-agar. The cells appeared as rods of various lengths and breadths measuring 0·75-0·9 : 1·5 μ

and 0·9 : 3 μ. The bacterium was motile, the motility being produced by single terminal or by many peritrichous flagella; up to five were observed. On nutrient agar, the stroke was narrow, white, raised and lumpy, becoming dry and rough from the formation of small, lateral folds. Upon glucose-gelatine, the stroke spread laterally as a broad, corrugated, white, moist-glistening growth with an amœboid tendency. The culture spread over the greater part of the slope, and in time became depressed and finally liquefied the medium. The stab in the same medium grew in a pronounced tubercular fashion, the lateral outgrowths measuring up to 3 mm. The nail-head spread over the whole surface as a depressed, corrugated, white film which was partly rough and partly glistening. A stratiform liquefaction eventually set in. Bouillon developed a strong white film and became turbid, with a white sediment. The indol reaction was obtained and nitrates were reduced to nitrites. Upon potato the growth was dry and scanty, in colour dirty-white. Milk was slowly made viscous. A thick transparent slime was formed on levulose-asparagine-tannin-agar. The colonies on glucose-gelatine were waxy-white and either rounded or amœboid. The centre of the colony was raised, then looking towards the periphery came a depression, then a corrugated circle, and finally a smooth margin. The consistency was viscous. Microscopically, the centre appeared indefinite, then came a circle with dark spots, then a granular margin with radial markings. By the fourteenth day, the margin of the colony, and especially the amœboid outgrowths, showed a patchy structure as if the granules had collected in heaps, leaving clear spaces intervening.

There seemed to be little similarity between this organism and *Bact. metarabinum*, either the normal race or the race already described in this paper. The slime grew well on saccharose-potato-agar without tannin, and although rather stiff it could be removed without disturbing the agar surface. After being converted into the soluble form by the autoclave treatment, the gum behaved to reagents like arabin. Dehydration did not make it insoluble as in the case of metarabin; it dissolved in water, form-

ing a thickened mucilage. When boiled with 5 % sulphuric acid for an hour, the bulk of the gum was hydrolysed to reducing substances which readily yielded osazone fractions. These were found to consist of tarry impurity and galactosazone; no arabinosazone could be obtained. The remaining portion of the gum hydrolysed to the same sugar.

Already races of a bacterium which produced a galactan gum and which gave the reactions for arabin had been separated from the Sugar-Cane and from the Quince. The white race from the Sugar-Cane was different from this in its cultural characters, the chief differences being in the nature of the colonies and the stab in glucose-gelatine, its appearance on saccharose-potato-agar, the absence of gas production in glucose media, the slow liquefaction of the gelatine, and the larger size of the organism. As it does not appear to have been hitherto described, I suggest the name *Bacillus pseudarabinus ii.*, in order that the bacteria which produce approximately similar gums may have approximately similar names.

The three bacteria, viz., *Bac. levaniformans*, *Bact. metarabinum* and *Bac. pseudarabinus ii.*, were the only active gum-formers found in the branches that were examined. Since the natural gum was different from those formed by the bacteria, there is the probability that they did not directly produce the gum. At present it cannot be said that the host-plant can modify the gum once formed by these bacteria into another kind. We know that it can induce the bacterium to produce a gum of another solubility, and since it can do this it may be able to do a little more and alter the nature of the gum. Of the bacteria, *Bact. metarabinum* produces a gum most nearly allied to the natural exudate.

The possibility that the natural gum might be pectin led me to examine it in this light. Pectose* occurs in unripe fruits, etc., and is insoluble in water and alcohol, but is converted into the soluble modification known as pectin by boiling with water or dilute acids and by enzymes. The solution of pectin, like

* Morley and Muir's edition of Watts' Dictionary of Chemistry; Reynolds Green's The Soluble Ferments.

mucin and the gums, is rendered more viscous by the presence of albuminoids. Alcohol, barium hydrate, and the basic lead acetates coagulate it, while neutral lead acetate and tannin have no action. It is oxidised by dilute nitric acid to mucic acid. When boiled with dilute alkalis it is converted into pectic acid, which is insoluble in water, and which when boiled with dilute acids is converted to arabic acid, that is arabin. The arabic acid thus obtained is hydrolysed to pectinose (arabinose) and a little known organic acid [possibly one of O'Sullivan's galactan-geddic acids].

Had the Hakea gum been pectin, the digestion in the autoclave ought to have changed part of it at least into parapectin or metapectin, which are precipitated with neutral lead acetate and barium chloride respectively. As these reagents did not induce precipitation, the gum is probably not pectin. In view of the doubtful nature of the gum, I resolved to boil it first with dilute alkali, then with dilute acid, and to test the products to see if any information could be gained respecting its possible affinities with pectin. Accordingly pieces of gum were boiled under an aerial condenser with 1 % sodium hydrate for two hours. It did not appear to dissolve, but remained as slightly swollen opalescent lumps in the boiling alkali. The solution was filtered off and treated with alcohol, when a small precipitate settled out. When treated with water and made faintly acid, this gave a ropy solution like the gum before treatment. Its reactions were identical with those given by the gum, so that the alkali had not altered this portion. The swollen gum, after being washed with water, was boiled with 1 % hydrochloric acid, in which it speedily dissolved. After an hour's boiling, the solution was neutralised and treated with alcohol, when a precipitate, which became flocculated with a drop of hydrochloric acid, was obtained. The alcoholic solution contained substances that reduced Fehling's solution. The precipitate, thrown down by the alcohol, dissolved readily in water, forming a comparatively slightly viscous solution like arabin. When the solution was made faintly acid and tested, coagula were obtained with ferric chloride and basic lead acetate.

Barium hydrate gave a slight stringy precipitate. Coagula were not obtained with Fehling's solution or with copper sulphate, although when the latter was followed by a trace of alkali a clot was formed. These are practically the reactions of arabin, and especially of dissolved metarabin. This is exactly what would have been expected from pectin, and supports the idea that the natural gum is that substance.

Unfortunately for this supposition, the hydrolysed products of the alkali-treated gum did not contain either arabinose or galactose, and from this we must conclude that the gum is not pectin. In testing this point, the digested gum was boiled for two hours with 1 % potassium hydrate, neutralised and precipitated with alcohol. The flocculent precipitate was boiled for three hours with 5 % sulphuric acid, neutralised and treated with alcohol, when a very small precipitate was thrown out. The solution, after elimination of the alcohol, was treated with phenylhydrazine acetate and the resulting osazone-like bodies purified with ether. They had much the same indefinite character of the substances previously obtained, and melted at about 120°, 140° and 175°. Neither arabinosazone nor galactosazone could be detected.

The conclusions to which this research has led are as follows:—

1. The gum of *Hakea saligna* is neither arabin, metarabin, nor pararabin. The hydrolytic products consist of reducing bodies that yield indefinite osazones and are probably akin to the furfuroïds of Cross, Bevan and Smith. It is not pectin, although it approaches this substance in some respects.

2. Of the bacteria occurring in the tissues of the plant, the most probable producer of the gum is one intermediate between *Bact. acaciæ* and its variety, *Bact. metarabinum*, but as we do not yet know that the host-plant can alter a gum once formed by a bacterium, it cannot be said that the gum is produced by this micro-organism.

3. Bacteria that produce galactan gums which behave to reagents like arabin are not uncommon. A second is described under the name of *Bacillus pseudarabinus ii.*, n.sp.